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OPTIMIZATION OF A-AMYLASE PRODUCTION FROM A LOCAL ISOLATE OF *BACILLUS LICHENIFORMIS* AND CHARACTERIZATION OF PURIFIED ENZYME

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ABSTRACT

A local isolate of Bacillus licheniformis which was isolated from potato, was the best producer of extracellular α-amylase. Optimum conditions ofenzyme productionwere:7g/l potato peels (carbon source), 23g/l wastes of fish (nitrogen source), 1.5g/l $MgSO_4.H_2O$, 4g/l K_2HPO_4, 37°C, 60 hr and 1.5% volume of inoculum. The enzyme was purified by concentration with (50-90)% saturation of $(NH_4)_2SO_4$, ion exchange using DEAE sephadex A-50 and gel filtration using sephadex G-100. Activity of purified enzyme was progressed at 45°C and pH 7.0, while the enzyme showed high stability at a temperature range (0-60)°C and pH (6.0-9.0), also the enzyme was more stable with $CaCl_2$, $MnSO_4$and $MgSO_4$at 1 mM and 5 mM. M.W ofα-amylase was 49.5kDalton, kinetic constants K_m and V_{max} were 1.12 mg/ml and 106 U/ml respectively.

KEYWORDS: Bacillus licheniformis, Identification, α -Amylase, Production, Purification, Characterization

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Original Article

INTRODUCTION

Microbial enzymes are widely used in industrialprocesses,α-amylase is one of the most important industrial enzyme with many application such as brewing, baking, textiles, pharmaceuticals, starch processing and detergents (Sidhu *et al*, 1997).

α-amylase (1,4-α- D-glucan-glucan hydrolase, EC. 3.2.1.1)hydrolyzes the internalα- 1,4 linkages in starch in a random fashion leading to the formation of soluble maltodextrins, maltose and glucose (Gangadharan*et al*, 2009). Although amylases can be derived from several sources, including plants, animals and microorganisms, today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry (Gupta *et al*, 2003).

Most of microbial α- amylase is produced by filamentous fungi like *Aspergillus* and *Rhizopus* as well as bacteria, such as *Bacillus* (Pandey, 2005).*Bacillus subtilis*, *B. stearothermophilus*, *B.licheniformis* and *B.amyloliquefaciens* are good producersof thermostableα-amylase and these have been widely used for commercial production of the enzyme for various applications (Prakash and Jaiswal, 2009).

The production of bacterial α- amylase is depended on type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, metal ions, pH, temperature, time of incubation and thermo stability (Prescott and Dunn's, 1987).

The aim of the present study is to optimize the conditions ofα- amylase production by a local isolate of *B.licheniformis* by using submerged cultures and characterize this enzyme.

MATERIALS AND METHODS

Sources of Bacterial Isolates

Soil and potato which was bought from the Basra city markets.

1 g of each soil and potato chopped samples were transferred separately to test tubes containing 9 ml of sterilized dist. water and heated to 80°C /10 min, then cooled and transferred 0.1 ml of each sample to petri dish containing Nutrient agar. The samples were spread by L-shape. Petri dishes were incubated at 37°C/48 hr. The medium was sterilized previously in autoclave at 121°C, 15 bar/inch^2/ 15 min before using.

Qualitative Screening of Isolate for Starch Hydrolysis: Using N.A with starch (Harly and Prescott,2002).

Biochemical Test for Identification of *Bacillus Licheniformis* (Harly and Prescott, 2002; Barrow and Feltham,2003; Logan andDeVos,2009).

Preparation of Inoculum: Using Luria- Bertani broth medium which composed of (g/l): peptone, 10, yeast extract, 5.0 and NaCl, 10.0, incubated overnight at 37°C in a rotary shaker at 150 rpm (Božić,*et al*, 2011).

α-amylase production: Using the medium, whichcomposed of (g/l): soluble starch, 5.0, peptone, 20.0, $MgSO_4.H_2O$, 1.0 and K_2HPO_4, 3.0. The pH of medium was adjusted to 7.0. Cultivation was maintained at 37°Cwith agitation at 150 rpm/24 hr (Božić,*et al*, 2011).

Optimization of α- Amylase Production

- **Carbon Source:** Soluble starch, potato peels, damaged rice, damaged unripe dates and wheat bran.
- **Quantity of Potato Peels:** (3, 5, 7, 9) g/l.
- **Nitrogen Source:** Peptone, tryptone, yeast extract, urea and wastes of fishes.
- **Quantity of Wastes of Fishes:** (17, 20, 23, 26) g/l.

Each wastes which were used in production media such as potato peels, rice, unripe dates, wheat bran and wastes of fishes were dried at 100°Cthen crushed in mortar to make small particles or powders.

- **$MgSO_4.H_2O$:** (0.5, 1.0, 1.5, 2.0) g/l. (Akcan*et al*.,2011)
- **K_2HPO_4:** (2.0, 3.0, 4.0, 6.0) g//l.
- **Temperature:** (25, 30, 35, 40, 45) °C. (Akcan*et al*.,2012)
- **Initial pH:** (5.5, 6.0, 6.5, 7.0, 7.5).
- **Incubation Period:** (12, 24, 36, 48, 60, 72, 84) hr.
- **Volume of Inoculum:** (0.5, 1.0, 1.5, 2.0) %

Extraction of α-Amylase:

The raw extract of α-amylase in the production media was separated by cold centrifugation at 6000 rpm/30 min/4°C (Sani *et al*, 2014).

Enzyme Assay: Using method of (Yang *et al*, 2003)

α-amylase activity determined by measuring the release of reducing sugars from soluble starch. The reaction mixture contained 0.5 ml of crude enzyme and 1 ml of sodium phosphate buffer (pH 7.0), then 1 %soluble starch was added and incubated at 25°C /10 min. The amount of reducing sugars was determined by the addition of 2, 3-dinitrosalicylic acid, followed by boiling for 10 min to develop color. The absorbance of the mixture was measured at 540 nm, the standard curve of reducing sugars with D- glucose.

One unit of enzyme activity was defined as the amount releasing reducing sugars equivalent to 1µ mole glucose per min under the assay conditions.

Protein Content Determination: Using method of Lowry *et al*, (1951).

Purification of α- Amylase

- **Concentration by Ammonium Sulphate (50-90) % Saturation:** At the beginning, the raw extract (crude enzyme) was saturated with 50% $(NH_4)_2SO_4$, then the precipitate was removed by centrifugation at 20000 rpm/15 min/4°C. Supernatant was raised to 70% saturation, the precipitate was removedby centrifugation at 20000 rpm/15 min/4°C. Supernatant was raised to 90%, thenthe precipitate was removedby centrifugation at 20000 rpm/15 min/4°C. The resulting supernatants were subjected to enzyme assay (Yandri*et al*, 2007).
- Dialysis for overnight at 4°C against 20 mM Na_2HPO_4 (pH 7.0).
- Ion exchange using DEAE-sephadex A-50 with a gradient salting (0-0.5) M of NaCl and 20 mM Na_2HPO_4 (pH 7.0). The column (1.5x25) cm, the fraction volume 3ml.
- Gel filtration using sephadex G-100 and 20 mM Na_2HPO_4 (pH 7.0).The column (2.5x75) cm, the fraction volume 3ml.

Characterization of Purified Alpha Amylase

- **Effect of Temperature Onα-Amylase Activity:** (15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70).
- **Effect of pHon α-Amylase Activity:** (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0).
- **The Thermo Stability of α-Amylase:** (0.0, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75).
- **Effect of pHon α-Amylasestability:** (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0).
- **Effect of Ion Metals and Chelating Compounds Onα-Amylase Activity:** $MgSO_4$, $FeCl_2$, $CaCl_2$, $MnSO_4$, urea, EDTA (1, 5) mM.
- The kinetics, K_m and V_{max} ofα-amylase were determined,according to the method of Lineweaver Burk reciprocal plot (Segel,1976).
- Estimation of molecular weight: Using polyacrylamide gel electrophoresis with SDS by method of Laemmli,(1970) in Garfin, (1990).

RESULTS AND DISCUSSIONS

Colonies of isolates were appeared with white or creamy color, spread without arranged surround. The cells were

G^+, rods, motile and had spherical spore, so the results showed that isolates belong to genus *Bacillus*and this agrees with Logan and DeVos,(2009).

Table 1 and 2 showed the physiological properties of isolates, they were positive in starch hydrolysis, casein hydrolysis, catalase, oxidase, gelatin liquefaction, nitrate reduction, citrate utilization also fermented glucose, trehalose, sucrose and arabinose. They were aerobic but can grow anaerobically, grow in 7% NaCl, also in pH 5.5, 8.0 and no growth at 60°C.These results indicated that isolates were *B. licheniformis*, so that agrees with Logan and DeVos,(2009) and Gordon *et al*,(1973)**.** *B. licheniformis*which was isolated from potato was the best producer ofα-amylase**,** this is according to potato is a starchy material which enhanced bacteria to produce amylases.

Table 1: Biochemical Test of *Bacillus licheniforms* Isolated from Potato

Test	Result	Test	Result	Test	Result
Gram staining	+	Citrate utilization	+	Vogasprokaur	+
Motility	+	Indol production	+	Gas production	w
Spore forming	+	Phenyl alanine	-	Growth in 5%Nacl	+
Anaerobic growth	+	Oxidase production	+	Growth in 7%Nacl	+
Starch hydrolysis	+	Catalase production	+	Growth in pH 5.5	+
Glatin hydrolysis	w	Nitrate reduction	+	Growth in pH 8	+
				Growth in 55 c̊	w
				Growth in 60 c̊	+

(+) =positive, (-) = negative, (w) = weak

Table 2: Carbohydrate Fermentation by *B. licheniformis* Isolated from Potato

Sugar	Result	Sugar	Result	Sugar	Result	Sugar	Result
Glucose	+	Maltose	+	Xylose	+	Sorbose	-
Fructose	+	Trehalose	+	Lactose	-	Sorbitol	-
Galactose	+	Mannose	+	Raffinose	-	Inulin	-
Arabinose	+	Dextrin	+	Melebiose	-		
Sucrose	+	Mannitol	+	Cellibiose	-		

(+) =positive, (-) = negative

Optimization of production of α-amylasecan be displayedin tab (3) and (4) which showed that potato peels was the best carbon source at 7g/l, because of starch content which enhanced production of α-amylase. Potato peels are food wastes and very useful in many biotechnological processes with no coast, furthermore it is friend toenvironment. The results were compared withBožić,*et al*,(2011), they found that 0.5% starch was the best carbon source in production of α-amylase produced by *B.subtiis*, also Krishnan and Chandra,(1983) found that less than 1% starch was the best carbon source for productionof α-amylase by *B.licheniformis*, and Akcan*et al*,(2011) used Luara broth toobtain high productivity of α-amylase.

Table 3: Effect of Carbon Source on Production of α-Amylase by of *B. licheniformis* isolated from Potato

Carbon Source	Activity u/ml
Soluble starch	570.305
Potato starch	595.260
Damaged rice	576.455
Damaged unripe dates	310.150
Wheat bran	325.340

Table 4: Effect of Quantity of Potato Peels on Production α-Amylase by *B. licheniformis* Isolated from Potato

Potato Peals (g/l)	Activity (u/ml)
3	460.375
5	595.760
7	687.300
9	575.450

Table 5 and 6 appeared that wastes of fishes were the best nitrogen source at 23g/l, therefore biotechnology can depends on such materialsas alternatives todecrease coast and pollution**.**When compared withother researchers, such as Božić,*et al*,(2011) found 2% peptone was the best nitrogensource, butAkcan*et al*,(2011) found casein was the best nitrogensource.

Table 5: Effect of Nitrogen Source on Production α-Amylase by *B. Licheniformis* Isolated from Potato

Nitrogen Source	Activity u/ml
Peptone	570.305
Tryptone	520.250
Yeast extract	495.487
Urea	378.55
Waste of fishes	595.760

Table 6: Effect of Quantity of Wastes of Fishes on Production α-Amylase by *B. licheniformis* Isolated from Potato

Wastes of Fishes g/l	Activity u/ml
17	420.375
20	595.760
23	610.240
26	530.756

Figure 1, 2**,** 3 and 4 revealed the best conditions were 37°C, pH 7.2, 60hr and 1.5%volume of inoculum.The present study agrees with El-Tayeb, *et al.,* (2007) andHaq, *et al.,* (2010), they found that 37°C was the best temperature, however it did not agree withAkcan*et al*,(2011), they found 72hr was the best incubation period and Haq, *et al.,* (2010) found the volume of inoculum 8% / 48 hr was the best in production of α-amylase from mutant *B. amyloliquefaciens.*

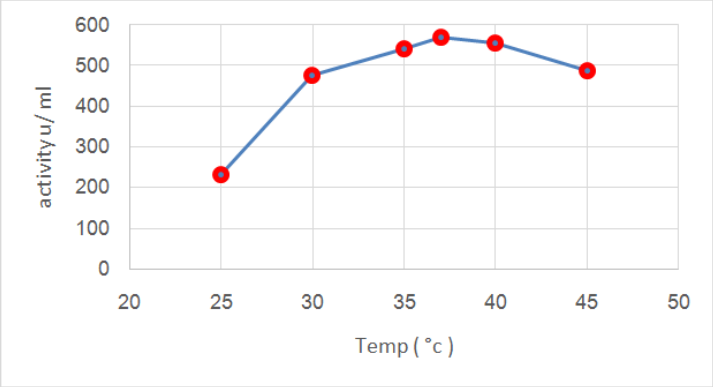

Figure 1: Effect of Temperature on Production of α-Amylase by Alocal Isolate of *B. licheniformis*

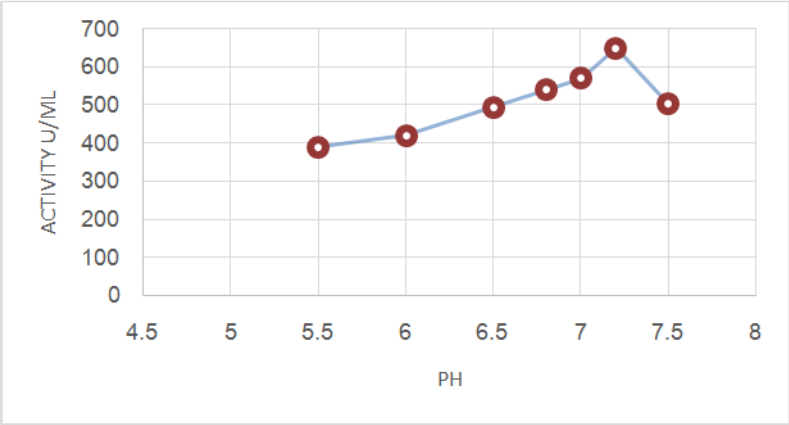

Figure 2: Effect of pH on Production of α-Amylase by Alocal Isolate of *B. licheniformis*

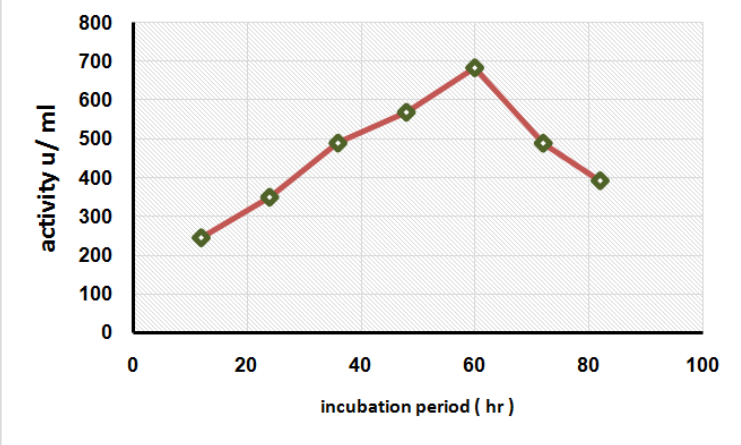

Figure 3: Effect of Incubation Period on Production of α-Amylase by Alocal isolate of *B. licheniformis*

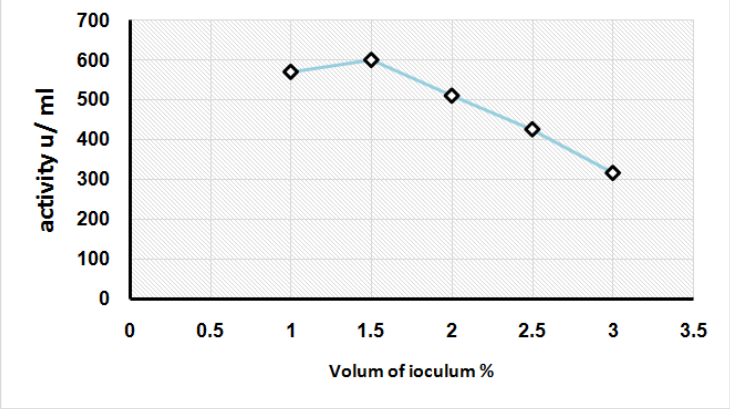

Figure 4: Effect of Volume of Inoculum on Production of α-Amylase by a Local Isolate of *B. licheniformis*

Table 7 and 8 showed that 1.5g/l $MgSO_4.H_2O$ and 3g/l K_2HPO_4 gave highest activity. This agrees withAkcan, *et al.*,(2012) in using $MgSO_4.H_2O$ to enhance production of enzyme from*B. subtilis.*

Table 7: Effect of $MgSO_4.H_2O$ on Production of α-Amylase by *B. licheniformis*

$MgSO_4.H_2O$ g/l	Activity u/ml
control	570.305
0.5	395.60
1.5	685.420
2	420.250

Table 8: Effect of K_2HPO_4 on Production of α-Amylase by *B. licheniformis*

K_2HPO_4 g/l	Activity u/ml
control	570.305
2	450.260
4	525.350
6	390.655

Table 9 revealed purification steps of α-amylase; The results indicated that the specific activity increased gradually for each step because activity was increased but protein was decreased. That agree with results of Sani *et al*,(2014), they studied partial purification ofα- amylase isolated from *B.subtalas*

Table 9: Purification Table of Extracted α-Amylase from *B. licheniformis*

Purification Step	Volume (ml)	Activity (u/ml)	Protein (mg)	Specific Activity (u/mg)	Total Activity (u/mg)	Yield %	Purifica-tion Fold (x)
Crude enzyme	350	685.430	0.521	1315.60	239900.5	100	1
Fractionation wit (NH4)2SO4	110	1020.504	0.178	5733.17	112255.44	46.79	4.35
DEAE sephadex A-50	46	872.531	0.093	9382.05	40136.426	16.73	7.13
Gel filtration G-100	40	825.640	0.032	25801.25	33025.6	13.76	19.61

Figure 5 appeared three protein peeksat ion exchange using DEAE sephadex-50,one peek in the first stage and two in the elution stage using graduate salting (0-0.5)M NaCl and only the third peek had enzymatic activity

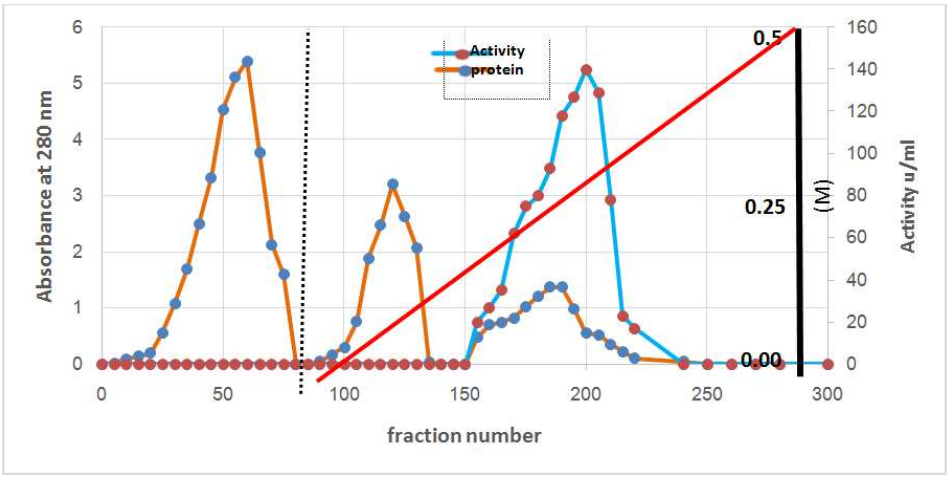

Figure 5: Ion Exchange for α-Amylase using DEAE-Sephadex A-50 with20 mM Na_2HPO_4 (pH 7.0) and (0-0.5) M of NaCl. The Column (1.5x25) cm, the Fraction Volume 3ml

Figure 6 appeared two protein peeks at gel filtration using sephadex G-100 and only the second had enzymatic activity

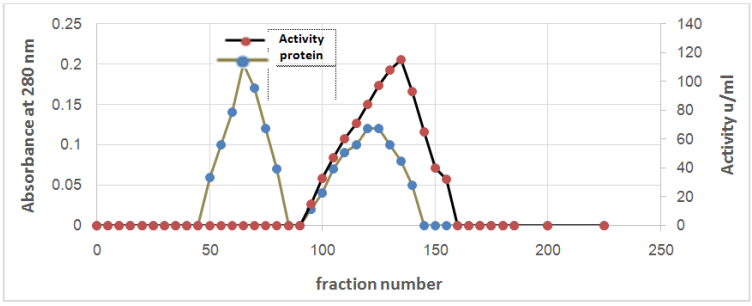

Figure 6: Gel Filtration for α-Amylase using Sephadex g-100 and 20 mm na_2hpo_4 (ph 7.0)، the Column (2.5x75) cm, the Fraction Volume 3ml

Figure 7 showed the best temperature for activity of purifiedα- amylase was 45°C.That was because Bacillus in general is thermoduric bacteria, thus this enzyme can be used in food and detergents industries. This agrees with Sani *et al*,(2014) but they found the enzyme was more thermal (60°C).

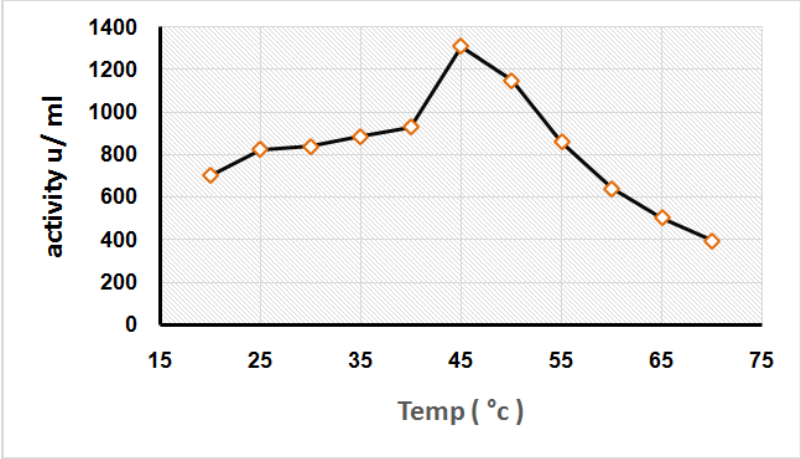

Figure 7: Effect of Temperature on Activity of Purified α-Amylase Produced by Alocal Isolate of *B. licheniformis*

Figure 8 appeared the best pH for activity of purified α-amylase was 7.0, this means that the enzyme works in neutral conditions and this is asymptotic to best pH of the enzyme of *B. subtilis* (pH 7.0) (Sani *et al*, 2014).

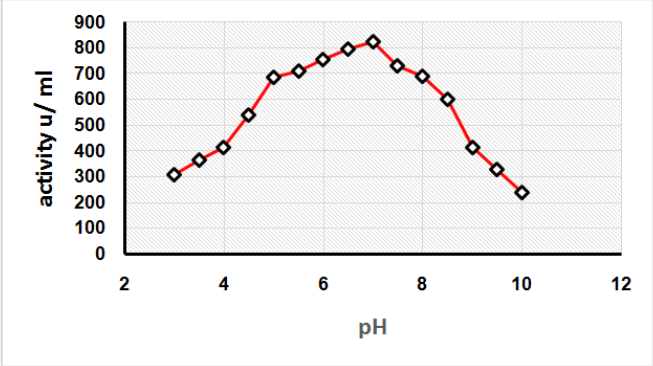

Figure 8: Effect of pH on Activity of Purified α-Amylase Produced by Alocal Isolate of *B. licheniformis*

Figure 9 showed that α-amylase was more stable at a range (0-70)°C, but Demirkan,(2011) found α- amylase was thermostable (70% at 50°C for 4 hr).

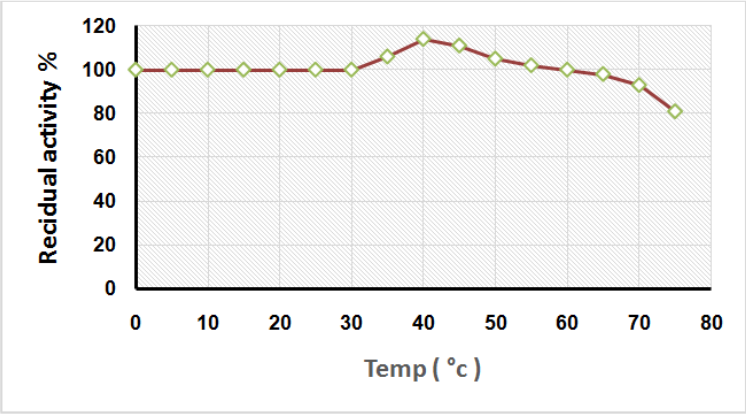

Figure 9: Effect of Temperature on Stability of Purified α-Amylase Produced by Alocal Isolate of *B. Licheniformis*

Figure 10 indicated thatα- amylase had high stability in neutral and alkaline pH (6.5, 7.0, 7.5, 8.0, 8.5), nonetheless Demirkan,(2011)found the enzyme produced by a mutant strain of *B.subtilis* was stable 85% at pH 8.0 and 33% at 5.0.

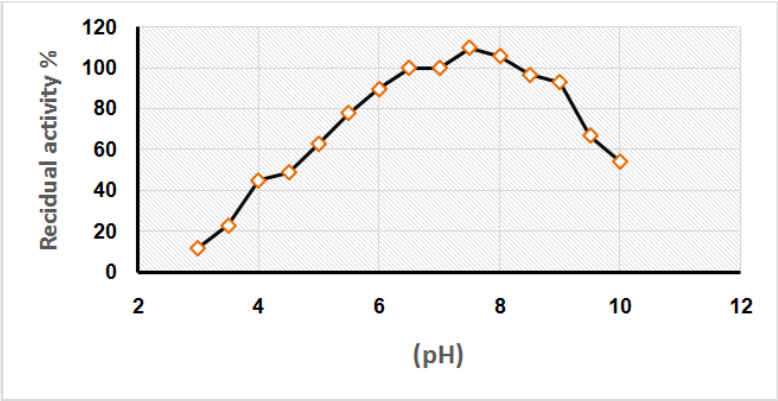

Figure 10: Effect of pH on Stability of Purified α-Amylase Produced by a Local Isolate of *B. licheniformis*

Table 10 appeared that purifiedα-amylase was more stable with $CaCl_2$,$MnSO_4$ and $MgSO_4$, also urea andEDTA in both 1mM and 5mM. |The result revealed that some ionswere affected especially Ca^{++} which is independent α-amylase (Carvalho, *et al.*, (2008).

Table 10: Effect of Metal Ions and Compounds on Activity of Purified α-Amylase

Metals and Compounds	Residual Activity %	
	1 Mm	5 Mm
Non	100	100
$MgSO_4.H_2o$	113	95
$FeCl_2$	94	0
$CaCl_2$	137	75
$MnSO_4$	120	92
Urea	103	48
EDTA	105	60

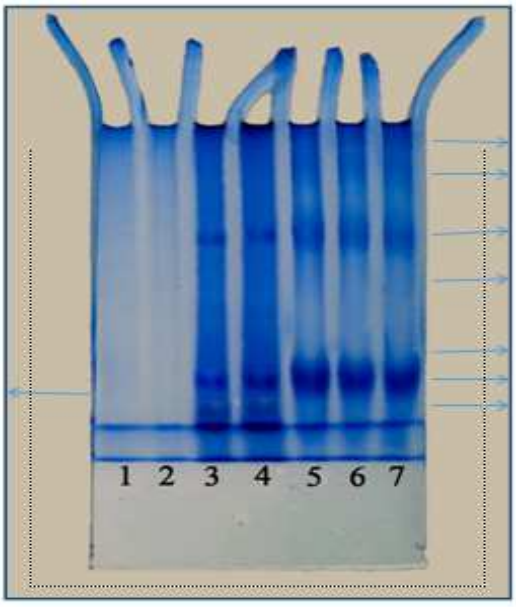

Figure 11: Estimation of Molecular Weight of Purified α-Amylase using PAGE-SDS. 1,2 α-Amylase 3,4,5,6,7 Standardproteins from up:Tyrosine hydroxylase 225 kD, Myosin 200 kD,IgG 150 kD, β-Galactosidase116.25 kD, Phosphorylase b 97.4 kD, Serum albumin 66.2 kD, Ovalbumin 45 kD

CONCLUSIONS

Figure 11showed thatα-amylase had one band with a M.W 49.5kD,this result do not agree with Demirkan,(2011).He found that M.W of α-amylase was 56kD because he used *Bacillus subtilis*asα-amylase producer.The kinetic constants.K_m and V_{max} of amylase were calculated from Michaelismenton equation and they were 1,12 mg/ml and 106 U/ml respectively.

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